

**ADDIS ABABA UNIVERSITY  
SCHOOL OF GRADUATE STUDIES**

**MOSQUITO LARVICIDAL *BACILLUS* STRAINS  
FROM ETHIOPIA**

**AKLILU SEYOUM**

**APRIL, 1996**

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FROM ETHIOPIA**

**A THESIS PRESENTED TO THE SCHOOL OF GRADUATE STUDIES  
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**IN PARTIAL FULFILLMENT OF THE DEGREE OF  
MASTER OF SCIENCE IN BIOLOGY**

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## ABSTRACT

Four strains of larvicidal bacilli isolated from soil and dead mosquito larvae in Ethiopia and two international standard strains, *Bacillus thuringiensis* var. *israelensis*, IPS-82 and *Bacillus sphaericus*, SPH-88, were investigated for their larvicidal potential using larvae of the mosquito species, *Culex quinquefasciatus* and *Anopheles arabiensis*. Among the four media types selected for cultivation of the strains, medium-2 and medium-3 were found suitable to enhance the toxicity of the Ethiopian strains. Moreover, culture incubation temperature and aeration rate were found to affect the larvicidal potential of the Ethiopian strains. The larvicidal efficacy ( $LC_{50}$  values) of the Ethiopian strains ranged from 0.0086 mg/l for *Bacillus* NA-82 on second instar larvae of *Cx. quinquefasciatus* to 221.6 mg/l for *Bacillus* NA-6 on third instar larvae of *An. arabiensis*. IPS-82 showed the highest larvicidal efficacy with  $LC_{50}$  value of 0.0010 mg/l against second instar larvae of *An. arabiensis*. Two of the Ethiopian strains and IPS-82 showed a higher toxicity on larvae of *An. arabiensis* than on *Cx. quinquefasciatus*. All Ethiopian strains have high degree of morphological and biochemical similarities to the reference strain of *B. thuringiensis* var. *israelensis* (IPS-82) and have been taxonomically classified as strains of *B. thuringiensis*. Moreover, serological characterization revealed that two of the Ethiopian strains, *Bacillus* NA-6 and *Bacillus* NA-71 are identical to *B. thuringiensis* var. *seoulensis*. The other two strains, *Bacillus* NA-10 and *Bacillus* NA-82 showed low level of titers to two of the type antisera tested and thus are possibly new serotypes of *B. thuringiensis*.

## 1 INTRODUCTION

Vector-borne diseases are among the major public health problems in developing countries. Mosquitoes are vectors of a variety of human diseases such as malaria, filariasis, yellow fever and dengue fever. The control of vector populations to limit the spread of diseases has been targeted as an area of special importance by the World Health Organization (WHO, 1983).

Over the last 45 years chemical insecticides have been used for the control of vector-borne diseases (Porter *et al.*, 1993). Unfortunately, more than 200 species of medically important arthropods have become resistant to many contemporary insecticides (WHO, 1993a). In addition, the long-term harmful effects of powerful chemicals to non-target organisms as well as the concern about the accumulation of pesticides in the environment has highlighted the need to develop alternative control measures. A promising alternative is the biological control of insect vectors of disease.

For nearly 30 years, *Bacillus thuringiensis* strains have been successfully used as biopesticides, mostly against agriculturally important caterpillar pests (Porter *et al.*, 1993). Efforts have been made to control insect vectors, particularly mosquitoes, with microbial control agents which are believed to have safety to the environment and not affecting non-target organisms. Among the microbial control agents of mosquito vectors of disease, *Bacillus thuringiensis* var. *israelensis* is the most successful (Goldberg and Margalit, 1977) and has been used in mosquito and blackfly control programmes (WHO, 1993a). Many strains of *Bacillus sphaericus* are also potent larvicides of mosquito species (Singer, 1979). Other promising bacterial species such

as *Clostridium bifermentans* var. *malaysia* and *Bacillus thuringiensis* var. *medellin* are under investigation (WHO, 1993b).

The major limitations of natural strains of *B. thuringiensis* var. *israelensis* and *B. sphaericus* are lack of persistence in mosquito breeding habitats due to rapid settling of the spore-crystal complexes (Davidson *et al.*, 1984; Ohana *et al.*, 1987), and narrow host range compared with that of chemical insecticides. The settling out problem is considered as the main obstacle for the control of vectors of malaria, that are surface feeders in their habit.

Though, improvements in potency, host range and persistence of both strains by genetic engineering and formulation technology are very essential, the search for new wild strains with higher potency, longer persistence and wider host range than those strains currently in use is also necessary for cost effective application in mosquito control programmes. Screening samples from different environments may yield larvicidal *Bacillus* strains with broader host ranges of new specificities. The host range of strains used commercially can be expanded through the introduction of new crystal protein genes. It was also recommended by the World Health Organization to continue the search for, isolation and evaluation of entomopathogens against a broad spectrum of mosquitoes and other vectors especially in areas where such a search has not been carried out before (WHO, 1993b). Moreover, mosquito resistance to *B. sphaericus* in the field and the possible appearance of resistance to *B. thuringiensis* var. *israelensis* (Lee *et al.*, 1994) emphasized the need to search for and characterize new strains which differ in potency, in their toxins or in their modes of action, from those currently in use. In

general, the availability of large numbers of diverse larvicidal strains may enable better management of resistance, broaden the host range of microbial control agents and increase their persistence in the field.

In Ethiopia, a preliminary screening for *Bacillus* species, which exhibit larvicidal effect against mosquito larvae, has been made through isolation from soils and dead mosquito larvae (Ali, 1993). However, characterization and identification was not complete. Moreover, the larvicidal efficacy on the different larval stages and mosquito species in comparison with the standard strains was not evaluated.

Therefore, the main objectives of this work were:

- a) to determine the larvicidal efficacy of isolated *Bacillus* species on mosquito vectors of disease and to compare their efficacy with the standard strains.
- b) to investigate the optimal conditions required for toxicity on mosquito larvae and
- c) to characterize the isolated *Bacillus* species using morphological, biochemical and serological tests.

## 2 LITERATURE REVIEW

### 2.1 Mosquito vectors of disease

Mosquitoes are the most familiar of all blood sucking insects. They fall into the nematoceran family, the Culicidae, which is divided into three subfamilies and 37 genera (Lehane, 1991). Members of the subfamilies Anophelinae and Culicinae

are important disease vectors, whereas the third subfamily Toxorhynchitinae, do not feed on blood and so are not disease vector.

In the subfamily Anophelinae there are about 400 species of *Anopheles* mosquitoes, but only some 60 species are vectors of malaria under natural conditions (Bruce-Chwatt, 1985). Some species of *Anopheles* mosquitoes are also known in the transmission of filarial parasites (*Wuchereria bancrofti* and *Brugia malayi*) and a few arboviruses.

The subfamily Culicinae, containing some 2808 species, are a taxonomically more complex group (Lehane, 1991). The medically important mosquitoes belong to the genera *Culex*, *Aedes*, *Mansonia*, and *Armigeres*. In urban areas *Culex quinquefasciatus*, which thrives in the polluted waters, is often the major vector of bancroftian filariasis and is also a biting nuisance species. Members of the genus *Aedes* are vicious biters and transmit yellow fever, dengue viruses and filariasis in several parts of the world.

Among a total of 42 *Anopheles* species recorded in Ethiopia, *Anopheles gambiae* complex is the main vector of malaria with the *Anopheles funestus* group, *Anopheles pharoensis* and *Anopheles nili* playing secondary roles (Geberemariam, 1988). Two sibling species of the *An. gambiae* complex (*Anopheles arabiensis* and *Anopheles quadriannulatus*) have been identified from Ethiopia (Zahar, 1985). *An. arabiensis* is an important vector of malaria and behaviorally it is an extremely plastic species; across its geographic range, exophilic and endophilic, anthropophilic and zoophilic forms are found (Lehane, 1991). *An. quadriannulatus* is a strongly zoophilic species which is not a vector of human disease. Over most of its range it

is exophilic, but in the highlands of Ethiopia, probably as an adaptation to the cold nights, it is endophilic (Lehane, 1991). The endophilic form may rarely feed on man but does not transmit human disease.

## 2.2 Entomopathogenic bacilli with mosquito control potential

The most important entomopathogens with potential to infect and kill mosquito larvae, belong to the spore-forming bacteria, the genus *Bacillus*. The most important bacterial entomopathogens are *Bacillus thuringiensis* Berliner and *Bacillus sphaericus* Neide.

### 2.2.1 *Bacillus thuringiensis* Berliner

The *B. thuringiensis* group of bacteria is composed of aerobic, gram positive endospore-forming rods. They are grown in culture in chains of 3-3.5  $\mu\text{m}$  long cells which have a cover of flagella all over the body (Weiser, 1991). Taxonomically, *B. thuringiensis* is closely related to *B. cereus* except the species *B. thuringiensis* is characterized by the production of parasporal crystal proteins associated with spore formation (Claus and Berkeley, 1986). The presence of parasporal crystals, specific flagellar (H) antigens, and entomopathogenic properties (except for some strains) distinguish clearly *B. thuringiensis* from many others species of the genus *Bacillus* (De berjack and Bonnefoi, 1968).

Together with a few biochemical characteristics, sub-specific division of *B.*

*thuringiensis* is based on the flagellar antigens of the vegetative cells (H-antigens) and the antigenic composition of the delta endotoxins (De berjack, 1982). The biochemical characteristics are not always strictly constant and that the identification of a strain must be based on all criteria (morphological, biochemical and serological). The flagellar agglutination technique provides the most specific, reliable and rapid means of assigning strains of *B. thuringiensis* into varieties (De berjack and Bonnefoi, 1968). Upto recently, 45 *B. thuringiensis* varieties have been identified based on their flagellar (H) antigens (Lecadet, personal communication).

*B. thuringiensis* var. *israelensis* also referred to as *B. thuringiensis* H-14 and *B.t.i* was first isolated in Israel (Goldberg and Margalit, 1977). Unlike many other members of the group, *B. thuringiensis* H-14 does not demonstrate useful activity against the larval stages of Lepidoptera but is highly pathogenic to larvae of blackflies and mosquito species (Nickerson, 1980).

The toxicity of *B. thuringiensis* H-14 to mosquito larvae is dependent upon parasporal crystals which are also called crystalline proteins, crystalline inclusions, parasporal bodies, parasporal inclusions and crystals. Upon ingestion by susceptible insect larvae, these crystalline inclusions are solubilized in the mid gut, releasing proteins called delta endotoxins. These proteins (protoxins) are activated by mid gut proteases, and the activated toxins interact with the larval mid gut epithelium causing a disruption in membrane integrity and ultimately leading to larval death (Gill *et al.*, 1992). Death probably results from disruption of the ionic regulation capacity of the mid-gut and the subsequent flow of toxic substances and ions into the haemocoel (Couch, 1981).

Another strain of *B. thuringiensis* var. *medellin* that showed toxicity towards *Culex quinquefasciatus*, *Aedes aegypti* and *Anopheles stephensi* larvae was isolated in Colombia (Orduz *et al.*, 1992). The parasporal crystals were spherical in shape and showed a great degree of similarity with those produced by the reference strain of *B. thuringiensis* H-14. Moreover, most of the biochemical features were identical to that of the reference strain. However, flagellar agglutination showed a specific H-30 antigen which made this strain to be described as a new serotype.

Some *B. thuringiensis* strains (eg. *B. thuringiensis* var. *kurstaki* HDI) kill mosquitoes as well as certain Lepidoptera larvae but there are other strains (eg. *B. thuringiensis* var. *kyushuensis*, *dermstadiensis*, and *morrisoni* PG-14) that are toxic primarily to members of the Diptera (eg. mosquitoes, blackflies, and midges) (Porter *et al.*, 1993). However, only in the case of *B. thuringiensis* H-14 field trials against mosquitoes have been carried out.

### 2.2.2 *Bacillus sphaericus* Neide

*B. sphaericus* is aerobic spore-forming bacterium widespread in soil and aquatic environments (Claus and Berkeley, 1986). It can grow in fine grey to brownish colonies in rods 2-3x1  $\mu\text{m}$  size which form spherical spores on the end of the rod. The spore protrudes from the sides of the rod and is 1-1.5  $\mu\text{m}$  in diameter (Weiser, 1991).

The insecticidal and non-insecticidal *B. sphaericus* have been classified by lytic bacteriophage typing (Yousten, 1984) and flagellar serotyping (De berjack *et al.*,

1985). The first strain of *B. sphaericus* pathogenic for mosquito larvae, strain "K" (Kellen *et al.*, 1965) was isolated from the moribund fourth instar larvae of *Culiseta incidens* in California, USA. Since then several mosquito pathogenic strains of this bacterium were isolated from dead mosquito larvae and non-mosquito origins.

The most widely studied mosquitocidal strains of *B. sphaericus* belong to serotypes H5a5b (eg. strains 1593 and 2362), H25 (eg. strain 2297) and H6 (eg. strain IAB59), which are all in the highly toxic group, and serotype H2 (eg. strain SSII-1), which falls in the low toxicity group (Porter *et al.*, 1993). Members of the highly toxic groups have toxic crystals which are protected within the exosporium, whereas strains with low toxicity lack the crystal (Baumann *et al.*, 1991). The mosquito larvicidal activity of the highly toxic strains of *B. sphaericus*, such as 2362, 2297 and 1593, is largely due to the presence of approximately equal amounts of antigenically distinct protein toxins produced during sporulation (Kalfon *et al.*, 1984).

### 2.3 Host ranges and field applications of larvicidal bacilli against mosquito species

Crystals produced by *B. thuringiensis* H-14 are toxic to dipteran larvae, including 72 species of mosquitoes (Culicidae) and 22 species of blackflies (Simuliidae) (Margalit and Dean, 1985). Data concerning the larvicidal efficacy of *B. thuringiensis* H-14 to different mosquito species showed that *Aedes* and *Culex* larvae are more susceptible than *Anopheles* larvae (De berjack, 1982). The lower susceptibility of *Anopheles* larvae is probably due to the particular surface feeding habits of the species, that could result in the lower uptake of the toxin. However,

Cheung and Hammock (1985) showed that formulations that enable floatation of toxic ingredients near the larval feeding zone could enhance the larvicidal activity of *B. thuringiensis* H-14 against *Anopheles* species. Moreover, Foo and Yap (1982) reported that the susceptibility of mosquito larvae to *B. thuringiensis* H-14 depends on the mosquito species within the genus.

*B. sphaericus* toxin has relatively higher activity towards larvae of *Culex*, variable toxicity to *Anopheles* depending on the species and is inactive on *Aedes* larvae (Ramoska *et al.*, 1977). Variations in susceptibility to the bacterium between strains of the same species of mosquitoes were also observed. This is probably due to differences in the nature of the target sites of the epithelial mid gut cells (Baumann *et al.*, 1991).

Moreover, Porter *et al.* (1993) have described three important factors which may govern the host range of toxins: i) differences in the larval gut which affect the solubility of the crystal complex, ii) the efficiency with which the protoxin is processed, and iii) the expression or availability of putative membrane receptors for the toxins in the gut of the insect.

Field evaluations showed that *B. thuringiensis* H-14 is very active and has a rapid specific action against mosquitoes even in harsh environments such as salt marshes (Purcell, 1981) and flood water (Mulla *et al.*, 1985). However, its rapid loss of residual effectiveness even at a very high rate of application in mosquito breeding habitats could result in *B. thuringiensis* H-14 producing less than 100% mosquito mortality in many habitats (Mulligan *et al.*, 1980).

Important attributes of *B. sphaericus* is its persistence in heavily polluted areas

which have promoted its use as a biocontrol agent (Priest, 1992). Entomogenous strains of *B. sphaericus* can be present in higher concentrations for significantly longer periods of time where mosquito larvae are present prior to inoculation (Des Rochers and Garcia, 1984). *B. sphaericus* may utilize the larval cadaver as a medium for growth and is released into the surrounding water as the cadaver disintegrates. On the other hand, there are also reports on the lack of residual activity of *B. sphaericus* due to the rapid settling of spores (Davidson, 1984).

#### **2.4 *Bacillus thuringiensis* for the control of agriculturally important pests**

Over the last 30 years, commercial use of *B. thuringiensis* pesticides has been largely restricted to a narrow range of lepidopteran (caterpillar) pests. In recent years, however, investigators have discovered *B. thuringiensis* pesticides with specificities for a much broader range of pests (Feitelson *et al.*, 1992).

The most promising area of use for *B. thuringiensis* preparations is on crops such as cabbage, lettuce, grapes, tomatoes and corn where the foliage or fruits is meant to be consumed (Angus, 1968). Pests of these crops feed throughout the entire growing period yet health regulations forbid the use of conventional insecticides close to the harvesting time. *B. thuringiensis* is recommended against defoliators where use of conventional insecticides is undesirable because of involvement of non target species. *B. thuringiensis* preparations are harmless to beneficial insects and vertebrates, thus permitting their unrestricted use up to the time of harvest (Scherrer *et al.*, 1973).

#### 2.4.1 *Bacillus thuringiensis* varieties against lepidopteran pests

*B. thuringiensis* is used against a wide range of lepidopterous defoliators in cotton, soybeans, grapes, tobacco, fruits, nuts, and vegetables (Couch and Ross, 1980). The two earliest known varieties of *B. thuringiensis* were isolates from silk worm (variety *sotto*) and from the Mediterranean flour moth (variety *thuringiensis*) (Angus, 1968). Since then several other *B. thuringiensis* varieties toxic to lepidopteran pests, were isolated from different parts of the world. The most widely studied and utilized strains belong to a variety *kurstaki* which was originally isolated from mass-reared colony of the pink bollworm, *Pectinophora gossypiella* (Dulmage, 1970a). *B. thuringiensis* var. *kurstaki* provided good and consistent field results and has become the standard control agent for cabbage looper and *Trichoplusia ni* (Couch and Ross, 1980). The progress made from the first commercial production with var. *thuringiensis* to the modern production with var. *kurstaki* indicates that the search for new strains can lead to still more potent toxin producing strains.

#### 2.4.2 *Bacillus thuringiensis* varieties against coleopteran pests

Many agronomically important pests are not lepidopterans (Herrnstadt *et al.*, 1986). Therefore, attempts were made to isolate strains of *B. thuringiensis* with activity against coleopteran pests. *B. thuringiensis* var. *tenebrionis* is the first coleopteran specific variety with activity against *Agelastica alni* and *Leptinotarta*

*decemlineata* isolated from larvae of *Tenebrio molitor* (Kreig *et al.*, 1983). Later, another strain of *B. thuringiensis* var. *san diego* that has toxic activity against coleopteran insects, colorado potato beetle and boll weevil, was isolated from larvae of *Tenebrio molitor* (Herrnstadt *et al.*, 1986). The isolation of both strains provided an opportunity for the development of new biocontrol agents against coleopteran pests.

## **2.5 Factors affecting toxin synthesis during growth (fermentation) of larvicidal bacilli**

The toxicity of larvicidal bacilli, that is the production of the toxic proteins differs several-fold within the same strain depending on the constituents of growth medium and growth conditions (Angus and Norris, 1968). Thus, it is essential to determine media composition and fermentation conditions for optimal larval toxin formation by larvicidal *Bacillus* species.

### **2.5.1 Media composition**

The media for industrial production of *B. thuringiensis* are basically composed of complex carbon and nitrogen sources. Starch and molasses are suitable carbon sources and protein rich material of plant and animal origin such as soy bean, corn steep liquor, or casein hydrolysates provide good and cheap nitrogen sources (Scherrer *et al.*, 1973).

### 2.5.2 Fermentation conditions

A marked difference in growth, sporulation, time at which toxin is produced during fermentation and level of toxin production at different fermentation conditions were observed for various *Bacillus* strains which exhibit mosquito larvicidal activity (Singer, 1979; Myers & Yousten, 1981; Yousten *et al.*, 1984). For instance, it was found that strain 2362 of *B. sphaericus* sporulated better and produced high level of toxin at higher temperature and in a medium with a higher protein content than did strain 1593 of the same species (Yousten *et al.*, 1984).

Investigations on the effect of temperature, dissolved oxygen, and pH on the growth, sporulation and toxin formation by strain 1593 (Yousten *et al.*, 1984), showed that *B. sphaericus* grew well from 25°C to 40°C, but sporulation and toxin synthesis were inhibited above 35°C. In addition, oxygen limitation during the period of toxin formation might be limiting the amount of toxin produced by this strain. It was also observed that at the pH near neutrality, the toxic activity of the bacteria was increased about ten fold.

According to Angus (1968), provided that sufficient nutrient is available, vegetative cell multiplication of *B. thuringiensis* can occur at low oxygen levels but sporulation and crystal production occur best with vigorous aeration of liquid cultures. Therefore, fermentation conditions for maximal toxin production should be investigated for new active strains of *Bacillus* with potential to kill mosquito larvae. Such investigations are essential to provide basic informations for small and large scale productions.

## 2.6 Factors affecting the efficacy of microbial control agents in practical mosquito control

The efficacy of microbial control agents was found to be influenced by many factors such as water characteristics of mosquito breeding sources (Mulla *et al.*, 1984), nutrient availability and ingestion rate of nutrients by mosquito larvae (Ramoska and Pacey, 1979), larval density (Darwazeh *et al.*, 1990), potency of formulations (Lacey, 1984), the larval stage and temperature (Wraight *et al.*, 1981).

### 2.6.1 Water characteristics of mosquito breeding habitats

Field evaluations showed that the efficacy of *B. sphaericus* against mosquito larvae is greatly affected by the nature and level of organic pollutants and that the efficacy is inversely proportional to the level of pollution (organic matter) in the water (Mulla *et al.*, 1984). It was also observed that *B. sphaericus* 2362 yielded good control of larvae upto 21 days post treatment in unpolluted and slightly polluted waters.

Similarly, simulated field studies with *B. thuringiensis* H-14 preparations against larvae of *Ae. aegypti* showed that the presence of soil constituents resulted in a lowering of larval mortality (Vanessen and Hambere, 1982).

### 2.6.2 Nutrient availability & ingestion rate by mosquito larvae

An increase in the amount of food available to mosquito larvae reduced the mortality at the specific dosage of *B. sphaericus* (Ramoska and Pacey, 1979). Suspended food particles may act as a competitor of the bacterium for consumption by mosquito larvae. Since the toxin produced by larvicidal bacilli acts as stomach poison, it should be sufficiently ingested to kill the mosquito larvae. Moreover, the feeding behavior including the zone of feeding (example, water surface, intermediate or bottom) can affect the efficacy of bacterial larvicides under field conditions (Sutherland and Khoo, 1987).

### 2.6.3 Larval density

An inverse relationship exists between larval density and the efficacy of *B. thuringiensis* H-14 (Darwazeh *et al.*, 1990). It was observed that, as the number of larvae increased in a constant volume of water, the dose of applied *B. thuringiensis* H-14 per larvae decreased and percentage larval mortality also decreased. This finding has practical implications in the field, where higher larval densities should dictate higher application rates.

### 2.6.4 Formulations

The major aims of formulations of microbial control agents are: increased

ease of handling, and enhanced activity via greater stability and maximized contact with the target insect (Lacey, 1984). Therefore, a formulation that enable floatation of spores and other toxic ingredients near the surface over a sustained period of time would enhance residual larvicidal activity against anopheline vectors of malaria.

### **2.6.5 Larval instars and temperature**

Late fourth instars of mosquito larvae that have a low feeding rate or have ceased feeding were not readily susceptible to *B. thuringiensis* H-14 (Mulla *et al.*, 1980) and *B. sphaericus* (Levy *et al.*, 1986).

Water temperature also had some effect on the activity of *B. thuringiensis* H-14 against mosquito larvae (Darwazeh *et al.*, 1990). It was observed that the second instars were found more susceptible at the lower temperatures than at the higher temperatures. However, the third and fourth instars showed a slight increase in tolerance at lower temperatures. It was also noted that a strong relationship between temperature and efficacy for *B. sphaericus* 2362. It has a somewhat higher activity level against larvae at higher temperatures using equivalent dosages.

## **2.7 Current status and future prospects on the development of microbial control agents of mosquito vectors**

Various formulations of *B. thuringiensis* H-14 are commercially available and are now widely used for vector control in many tropical countries, as well as against

nuisance mosquitoes and blackflies in Europe and North America. Another spore-forming bacterium, *B. sphaericus*, is now considered as the most promising organism for the control of *Culex* mosquitoes (WHO, 1993a).

The feasibility of using *B. thuringiensis* and *B. sphaericus* for mosquito control has been increased by advances in recombinant DNA technology. Some of the major achievements to improve the efficacy of mosquitocidal bacteria using recombinant DNA technology are the following:

a) All genetic toxin determinants for both *B. thuringiensis* H-14 and *B. sphaericus* have been cloned and characterized. Genes specifying crystal proteins in *B. thuringiensis* H-14 are located on plasmids (Priest, 1992). Four different Cry (crystal protein) genes, as well as the Cyt A (cytolytic crystal protein) gene were isolated from the same 72 MDa plasmid present in the strains of *B. thuringiensis* H-14 (Himeno *et al.*, 1985). The Cry IVA, Cry IVB, Cry IVC, and Cry IVD genes encode proteins with predicted molecular masses of 135, 128, 78 and 72 KDa, respectively.

These proteins assemble together with the 27 KDa Cyt A gene product, in ovoid crystal complexes (Hofte and Whitely, 1989). The toxin of *B. sphaericus* consists of two polypeptides with apparent molecular size of 43 and 56 kDa (measured by polyacrylamide gel electrophoresis) and both polypeptides seem to be required for larvicidal activity (Baumann *et al.*, 1987).

b) Suitable and stable plasmid vectors were identified and used for cloning and expression of *B. thuringiensis* H-14 toxin genes in *B. sphaericus* (Seyler and Yousten, 1991). The toxins produced by *B. thuringiensis* H-14 and *B. sphaericus* have different host range specificities. Moreover, *B. sphaericus* has the benefit of longer

persistence in mosquito breeding habitats. It is therefore of great advantage for mosquito control to combine the toxin genes from two microorganisms into one microorganism in order to widen the host range spectrum with longer persistence in mosquito breeding habitats.

c) It was also attempted to clone the genes encoding the toxins from *B. thuringiensis* H-14 and *B. sphaericus* into the broad host range plasmid pRK248 and the transfer of these genes for expression in *Caulobacter crescentus* CB15 (Thanabalu *et al.*, 1992). *Caulobacter* species are ubiquitous microorganisms residing in the upper regions of aquatic environments and therefore provide the potential for prolonged control by maintaining mosquitocidal toxins in larval feeding zones.

In another study, a mosquitocidal *Cyanobacterium* (normally exist at or near the water surface) also developed by introducing the mosquito toxic Cry IVD gene from *B. thuringiensis* H-14 into *Cyanobacterium aginenellum quadruplicatum* PR-6 (*Synechococcus* species, strain pcc 7002) (Murphy and Stevens, 1992). Larvicidal assays revealed that freshly hatched *Cx. pipiens* mosquito larvae readily ingested the transformed *cyanobacteria* and that the cells proved to be toxic to the larvae. These approaches provide possible solutions to the settling problem and may circumvent the need to develop costly floating formulations of bacteria.

All these major achievements provide new possibilities for increasing the efficacy of bacterial larvicides for vector control. The key factors are improved target toxicity, persistence and cost effective production and application. Genetic engineering offers the possibility of combining the best properties of *B. thuringiensis* H-14 and *B. sphaericus* within a single host.

Although, molecular genetics provide tools to improve the efficacy of microbial control agents; there is the need to search and characterize strains producing novel toxins for overcoming potential problems associated with resistance to existing toxins. To date there have been no reported cases of resistance development toward *B. thuringiensis* H-14, but mosquito resistance to *B. sphaericus* has been observed in the field (Lee *et al.*, 1994). However, resistance development has been demonstrated in the laboratory to toxins found in *B. thuringiensis* H-14 (Gill *et al.*, 1992).

Preliminary laboratory study on the development of mosquito larvae resistance to *B. thuringiensis* strains has been shown to be associated with a change or effect on the receptor sites of the mid gut epithelial cells (WHO, 1993b). Therefore, it seems that there is a potential for mosquito resistance to *B. thuringiensis* H-14 under field conditions. However, the rate at which acquired resistance develops will probably be lower than the rate for synthetic insecticides. An important factor that may have contribution to this is the low persistence of *B. thuringiensis* H-14 in the natural environment.

### 3 MATERIALS AND METHODS

#### 3.1 Bacterial strains and mosquito species

Larvicidal *Bacillus* strains used in this study coded as *Bacillus* NA-6, *Bacillus* NA-10, and *Bacillus* NA-71 were isolated from soil samples, while *Bacillus* NA-2 and *Bacillus* NA-82 from dead *Culex* larvae collected in Ethiopia (Ali, 1993). The strains were maintained by subculture on nutrient agar slants, at the Department of Biology, Addis Ababa University.

International standard strains of *B. thuringiensis* H-14 (IPS-82) and *B. sphaericus* (SPH-88) were obtained from the Institute of Pasteur, Paris, for comparison. These reference strains were used to compare the morphological and biochemical characteristics and also the larvicidal efficacy of the Ethiopian strains.

For the standard bioassay tests, colonies of *Culex quinquefasciatus* maintained at the insectary of the Institute of Pathobiology, Addis Ababa University were used. Identification of the species was confirmed using a key "Mosquitoes of the Ethiopian Region -I" (Hopkins, 1952). Larvae of *Anopheles arabiensis* were also obtained from colonies maintained at the insectary of the National Organization for the Control of Malaria and Other Vector-borne Diseases, Nazareth, Ethiopia. The cytotaxonomic identity of the colony maintained in Nazareth was established as *An. arabiensis* by Mekuria *et al.* (1991).

### 3.2 Preliminary tests

Preliminary tests on the larvicidal potential of all Ethiopian strains were done according to guidelines for isolation and screening of spore-forming bacteria possessing vector control potential (WHO, 1987). A loopful of bacterial colony grown on nutrient agar plates was transferred into 10 ml of sterile water in a test cup and mixed thoroughly. Ten 2<sup>nd</sup> instar *Culex* larvae were placed in the mixture. Test cups into which no bacteria were added served as control. Percentage larval mortality at 24 and 48 hr were recorded. All tests were carried out in duplicates, at three different times, and the mean percentage mortality was calculated for each strain.

### 3.3 Media compositions for cultivation of strains

In order to find an optimal medium for better efficacy of the strains four different types of media were selected.

#### 3.3.1 Medium 1 (M1)

This is a modified form of a medium used earlier for production of *B. thuringiensis* H-14 by De berjack (1982). It is composed of the following ingredients.

Glucose	10 g.
Cerelac	15 g.
Peptone	5 g.

Yeast extract	5 g.
$\text{KH}_2\text{PO}_4 \cdot 7\text{H}_2\text{O}$	0.1 g.
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5 g.
NaCl	3 g.
$\text{FeSO}_4$	0.1 g.
Distilled water	1 lit.
pH = 7.0	

### 3.3.2 Medium 2 (M2)

This culture medium is used earlier for cultivation of *B. sphaericus* by Yousten *et al.* (1984), and it is composed of the following ingredients.

Nutrient broth supplemented with yeast extract	13 g.
$\text{MnCl}_2$	0.06 g.
$\text{CaCl}_2$	0.07 g.
$\text{MgCl}_2$	0.3 g.
Distilled water	1 lit.
pH = 7.0	

### 3.3.3 Medium 3 (M3)

Medium 3 is used earlier for cultivation of *B. thuringiensis* by Scherrer *et al.* (1973), and it is composed of the following ingredients.

Glucose	10 g.
$(\text{NH}_4)_2\text{SO}_4$	2 g.

$K_2HPO_4 \cdot 3H_2O$	0.5 g.
$MgSO_4 \cdot 7H_2O$	0.2 g.
$MnSO_4 \cdot 4H_2O$	0.05 g.
$CaCl_2 \cdot 2H_2O$	0.08 g.
Yeast extract	2 g.
Distilled water	1 lit.
pH = 7.3	

#### 3.3.4 Medium 4 (M4)

Medium 4 was a modified form of a medium used earlier for local production of *B. thuringiensis* H-14 and *B. sphaericus* by Hertlein *et al.* (1981). It was composed of beef meat and wheat flour. 300 ml of distilled water was added to 30 g of beef meat. The mixture was blended by metal rod and decanted into 1000 ml of Erlenmeyer flask. The blended mixture was then placed in a boiling water bath for 1 hr and later filtered. In another flask 5 g of wheat flour was dissolved in 300 ml of distilled water and then filtered. Both preparations were then mixed at equal ratio to obtain the final medium (M4) and the pH was adjusted to 7.0.

All media types were sterilized at 121°C for 15 minutes before they were used for cultivation of the strains.

#### 3.4 Optimization of culture conditions

Standard inocula were prepared from all strains grown on nutrient agar slants.

One ml of the inoculum from each strain was used to inoculate a series of 250 ml Erlenmeyer flasks, each containing 50 ml of medium M1, M2, M3 and M4. The flasks were placed on a rotary shaker at 150 rpm and incubated for 72 hr at room temperature. In order to investigate the effect of aeration on larvicidal efficacy of the strains, cultures were also prepared and cultivated at 120 rpm using optimal media types at room temperature. To investigate optimal temperature for the larvicidal efficacy of the strains four groups of flasks were inoculated and each incubated at 25°C, 30°C, 35°C and 40°C using optimal media types and aeration level for 72 hr. Sporulation and crystal formation was monitored microscopically and the pH of the culture broth was adjusted to neutral before used for testing.

All culture preparations described above were investigated for their toxicity to *Culex* larvae. The efficacy of the strains were determined by adding 3 ml of whole bacterial culture suspensions into 27 ml of distilled water in a 100 ml beaker containing ten 2<sup>nd</sup> instar *Culex* larvae. Beakers into which no bacterial cultures were added served as control. All tests were carried out three times in duplicates and the mean percentage larval mortality at 48 hr was recorded. The relative toxicity was evaluated as percentage larval mortality of each culture suspension.

Media types and culture conditions that produced the highest larval mortality in 48 hr were selected for each strain and used to harvest bacterial biomass for the standard bioassay tests on laboratory reared mosquito species.

### 3.5 Assay material preparation

Four flasks, each containing 50 ml of optimal medium, were inoculated and incubated at optimal temperature and aeration levels of each strain in an incubator shaker for 72 hr. The final pH levels of cultures were adjusted to neutrality. The cultures were then centrifuged at 5000 rpm for 30 min. The supernatants were discarded and the residues were collected in penicillin flasks and then air-dried at room temperature, until constant weight was attained for each bacterial biomass.

### 3.6 Mosquito rearing

For the purpose of standard bioassay tests on identified mosquito species and instar levels, *Cx. quinquefasciatus* was reared at the insectary of the Institute of Pathobiology, Addis Ababa University.

Adult mosquitoes were placed in Baraud's mosquito cage, 30x30x30 cm. They were fed on 10% sugar solution soaked in cotton pad and placed on a pick dish. The cotton pad was renewed once a week. In addition, adult females were exposed to the human arm once a week to feed on blood. When the females were about to lay eggs, a small cup containing a small amount of water was placed in the cage. After oviposition, eggs were collected and transferred into larval rearing pans where hatching took place.

Larvae were reared in shallow pans, filled with water to a depth of

approximately 5 cm. The larvae were fed on larval food that was composed of "fafa", milk powder and dried yeast. Moulting of each of the four larval stages was routinely checked and categorized by the instar for the bioassay tests. Pupae were transferred from the rearing pans to plastic cups and kept in the cage until adults emerged.

### 3.7 Bioassay tests

Standard bioassay tests were performed for the two international standard strains (IPS-82 and SPH-88) and the four Ethiopian strains (*Bacillus* NA-6, *Bacillus* NA-10, *Bacillus* NA-71 and *Bacillus* NA-82). Target mosquito larvae were 2<sup>nd</sup>, 3<sup>rd</sup> and 4<sup>th</sup> instar *Cx. quinquefasciatus* and also 2<sup>nd</sup> and 3<sup>rd</sup> instar *An. arabiensis*. Fourth instar larvae of *An. arabiensis* was not included, due to pupation before 48 hr when they were exposed at low concentrations.

Bioassay tests were carried out according to the test procedure obtained from the WHO collaborating center for entomopathogenic *Bacillus* (Institute of Pasteur, Paris, France) (Lecadet, personal communication). However, assay concentrations of the standard and Ethiopian strains were adjusted based on the range-finding bioassay tests.

Fifty milligram of the standard powders (IPS-82 and SPH-88) and variable amounts of the test materials of the Ethiopian strains were placed in 20 ml penicillin flasks to which 10 ml of deionized water and 15 glass beads (6 mm diameter) were added. The suspensions were thoroughly homogenized by shaking

on Vortex mixer for few seconds.

Stock solutions of the standard strains were made in test tubes by serial dilution of the homogenates in deionized water and appropriate dilutions were prepared for the test against the different larval stages. The tests were done by adding known volumes from the appropriate dilutions into plastic cups containing 150 ml (for the standard strains) or 100 ml (for Ethiopian strains) deionized water. Five different concentrations of the test materials were prepared in duplicate for each assay. To each cup 20 mosquito larvae were added by Pasteur pipette. The control contained 20 mosquito larvae in 100 ml or 150 ml of deionized water.

Mortality was determined at 24 and 48 hr. It was based on counting of live larvae. The number of mosquito larvae dead was obtained by subtracting the total live larvae from the total exposed in each assay. The results for the 48 hr mortality were subjected to probit analysis on a computer program and the  $LC_{50}$  (lethal concentration that kills 50% of the total population) values were obtained at 95% confidence limits. The heterogeneity, in terms of the chi-squared test, was also tested. When control mortality exceeded 5%, the mortality rate of the treated groups were corrected according to Abbotts' formula (Swaroop, 1966). Test results with control mortality greater than 10% were discarded and repeated.

### 3.8 Larvicidal activity in natural water

*Bacillus* NA-82 and the two international standard strains were tested against field collected *Culex* larvae using water from the natural breeding sources.

Two hundred second instar *Culex* larvae that were collected from Kebena river, Addis Ababa, were added to small artificial concrete ponds. filled with 10 liter of water obtained from the same source. one mg, 0.09 mg and 0.01 mg biomass of each strain were applied to "experimental" ponds. Pond water containing 200 mosquito larvae without the test materials was used as control in each experiment. Each test was repeated two times on different dates.

Number of mosquito larvae dead after 24 and 48 hr were counted for both experimental and control ponds. The formula (Mulla *et al.*, 1985) was used in the calculation of the mean percentage reduction of larval populations:

$$(\text{Mean percentage reduction}) = 100 - \left( \frac{C_1}{T_1} \times \frac{T_2}{C_2} \right) 100, \text{ where}$$

$C_1$  = Mean number of mosquito larvae in control ponds before application of the test materials in "experimental" ponds.

$T_1$  = Mean number of mosquito larvae in "experimental" ponds before application of the test materials.

$T_2$  = Mean number of mosquito larvae in "experimental" ponds after application of the test materials.

$C_2$  = Mean number of mosquito larvae in control ponds after application of the test materials in "experimental" ponds.

P-values were calculated for the differences in the mean percentage reduction of larval populations in 24 and 48 hr. The pH of the water, the turbidity and the day time water temperature during the periods of experimentation were also recorded.

### 3.9 Morphological and biochemical characterizations

The morphology of bacterial cells from cultures grown on nutrient agar slants were observed under a light microscope. The shape of the spores, the type of sporangia and the presence or absence of parasporal crystals were recorded. The crystal staining method of Smirnoff (1962) was used for detection of the parasporal crystals.

Biochemical tests that were carried out for characterization of the strains are: production of catalase; Voges-Proskauer reaction; egg yolk reaction; growth at different concentrations of NaCl; acid production from carbohydrates (glucose, arabinose and mannitol); reduction of nitrate to nitrite; production of indole; production of dihydroxyacetone; deamination of phenylalanine; decomposition of casein; hydrolysis of starch; liquefaction of gelatin; and utilization of citrate and propionate.

Growth conditions at temperature 5°C, 20°C, 30°C, 40°C, 50°C and 65°C, growth conditions at pH less than 6 and greater than 7, and anaerobic growth conditions were also investigated. All biochemical tests were carried out according to Claus and Berkeley (1986).

For the biochemical confirmatory tests, standard strains of *B. thuringiensis* H-14 (IPS-82) and *B. sphaericus* (SPH-88) were used as reference strains and compared for their similarities and differences to the Ethiopian strains. Percentage similarities among all Ethiopian strains and the two standard strains were determined. This is based on simple matching similarity in which both shared negative and positive

results are regarded as similarities.

### 3.10 Serological characterization

All mosquito toxic Ethiopian strains that fulfilled the morphological and biochemical profiles for *B. thuringiensis* were serotyped. Slide and tube agglutination methods were employed according to Padua *et al.* (1984), using well motile bacteria and H-antisera of the 45 *B. thuringiensis* serotypes obtained kindly from the WHO collaborating center for entomopathogenic *Bacillus* (Institute of Pasteur, Paris, France).

#### 3.10.1 Enhancement of motility and H-antigen preparation

The motility of the four mosquito toxic *B. thuringiensis* strains was enhanced by passing them through tubes of semi-solid agar motility medium (beef extract, 3 g; peptone, 10 g; NaCl, 5 g; agar, 4 g; and distilled water 1000 ml).

Inocula were taken from the growth at the farthest point of migration which were then transferred to infusion broth. The broth cultures were incubated at 37°C for 24 hr. Microscopic observations were made by taking a loopful of culture suspensions on the slides to check their motility. Broth cultures of well motile and flagellated bacteria were inactivated using 0.6% formalin and incubated in a water bath at 37°C for 4 hr.

### 3.10.2 Slide and tube agglutination tests

For the slide agglutination tests, one drop of the flagellated bacterial suspension was mixed on a glass slide with one drop of the antiserum which had been diluted to 20 fold with saline. As a negative control, one drop of saline was mixed on a slide with a drop of bacterial suspension. Moreover, a drop of bacterial suspension of a reference strain, *B. thuringiensis* var. *israelensis* was mixed with its own H-antiserum to serve as a positive control. The slides were tilted back and forth for 1 minute and examined for possible agglutination, using low power microscope. The degree of slide agglutinations were graded as + + + +, + + +, + + and + to represent approximately 100%, 75%, 50% and 25%, respectively of the cells agglutinated.

Two fold serial dilutions of the antisera which had been originally diluted to 50 fold were prepared in saline for the tube agglutination in which 0.5 ml of each antiserum dilution was mixed with 0.5 ml of the suspension of formalin killed vegetative cells. Test tubes with bacterial suspension mixed with saline served as a negative control. Moreover, test tubes with culture suspension of the reference strain, *B. thuringiensis* var. *israelensis* mixed with antiserum dilutions of its own type served as positive control. All the tubes were incubated in a water bath at 37°C for 4 hr. All test tubes containing H-antigen and H-antiserum as well as the controls were examined for possible agglutinations by viewing the tubes under good light with the aid of magnifying lens. The agglutinin titer of the antiserum was expressed as the reciprocal of the greatest dilution giving visible agglutination.

## 4 RESULTS

## 4.1 Preliminary tests

Preliminary evaluation on the larvicidal potential of all Ethiopian strains against 2<sup>nd</sup> instar *Culex* larvae (Table 1) showed differences in their toxicity. The highest mortality is recorded for *Bacillus* NA-82 with 70% mortality in 48 hr. *Bacillus* NA-2 exhibited the lowest effectiveness with only 10% larval mortality in 48 hr.

Table 1. Mean percentage larval mortality at 24 and 48 hr obtained from strains grown on nutrient agar plates.

Code of strains	Mean percentage larval mortality (2 <sup>nd</sup> instar <i>Culex</i> larvae)	
	24 hr	48 hr
<i>Bacillus</i> NA-2	0	10
<i>Bacillus</i> NA-6	20	50
<i>Bacillus</i> NA-10	20	50
<i>Bacillus</i> NA-71	30	60
<i>Bacillus</i> NA-82	30	70
Control	0	0

All strains that showed percentage mortality of 50% and above at 48 hr were selected and used for further investigation. Thus *Bacillus* NA-2 that showed percentage mortality of 10% in 48 hr was excluded from further investigation due to its low efficacy.

#### 4.2 Optimal media types & culture conditions for larvicidal efficacy

The four media types used for cultivation of the Ethiopian strains showed differences in their potential to support toxin production. This can be shown from the differences in percentage larval mortality obtained from strains grown on different media types. Similarly, there were some differences on the larvicidal efficacy of each strain grown at different aeration level and temperature. The natural mortality rate was 0% in all control test cups.

##### 4.2.1 The effect of media composition on the larvicidal efficacy of strains

Investigation on the effect of media composition on the larvicidal efficacy of the strains (Fig. 1) showed in general that different larvicidal strains have different media composition as their optima. Medium-2 (M-2) is the best medium type for *Bacillus* NA-10 and *Bacillus* NA-82 with 80% and 100% larval mortality, respectively. For *Bacillus* NA-6 and *Bacillus* NA-71 medium-3 (M-3) is found to be an optimal medium with 70% and 90% larval mortality, respectively. Percentage larval mortality recorded for all Ethiopian strains when they were grown on medium-1 (M-1) and medium-4 (M-4) were found to be less than 50%, except for *Bacillus* NA-71 when it was cultivated from medium-1

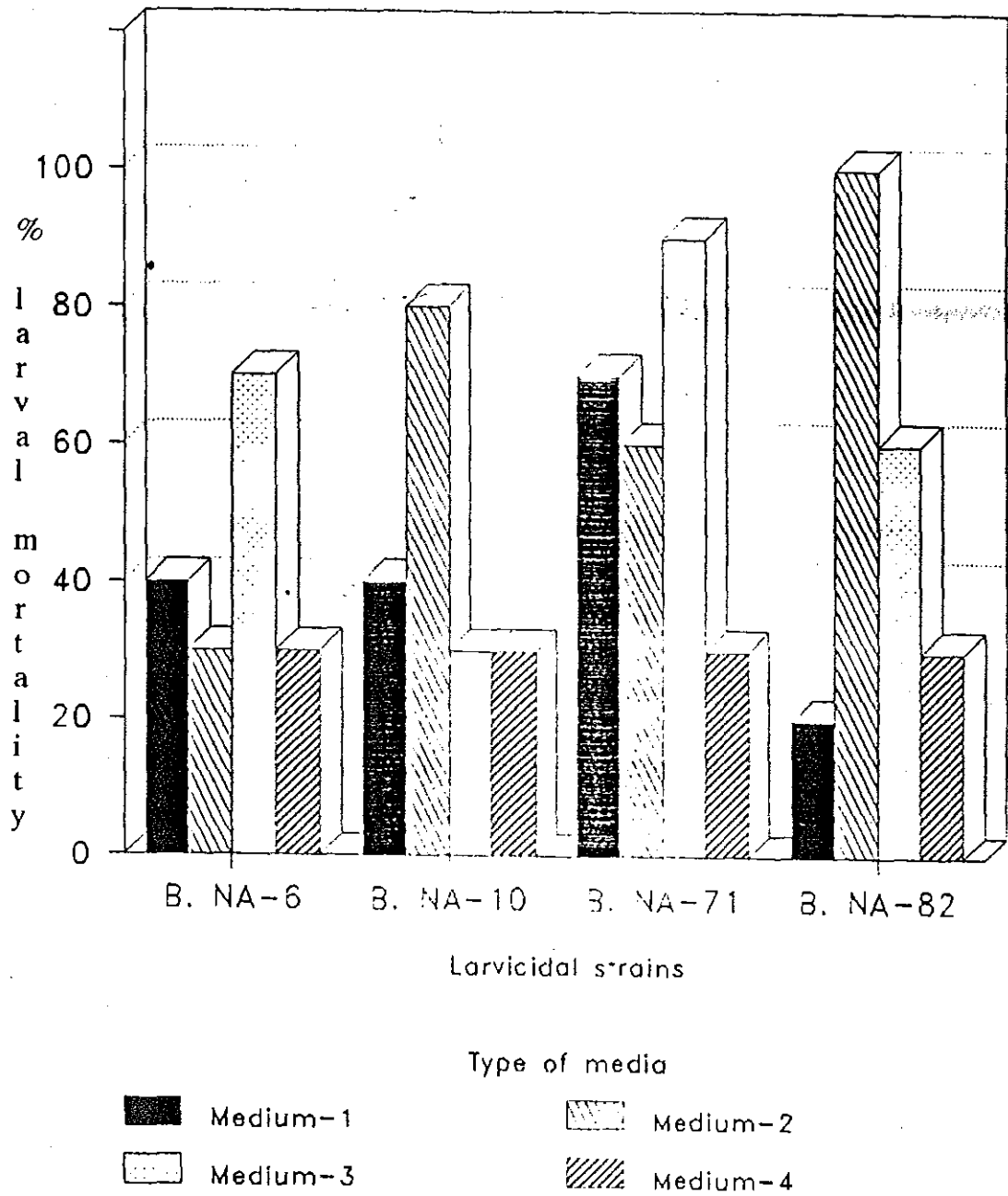


Fig.1. Effect of media composition on the larvicidal efficacy of strains.

(70% larval mortality).

#### 4.2.2 Effect of aeration on the larvicidal efficacy of strains

The effect of aeration level on the efficacy of strains (Fig. 2), showed better toxicity for each strain when cultivated at 150 rpm than at 120 rpm. *Bacillus* NA-6 showed the highest difference in toxicity when it was cultivated at 120 (20% larval mortality) and 150 rpm (70% larval mortality). The effect of aeration was found to be minimum on the toxicity of *Bacillus* NA-10 than the other strains. *Bacillus* NA-10 showed the least difference, 70% and 80% larval mortality when it was cultivated at 120 and 150 rpm, respectively.

#### 4.2.3 The effect of cultivation temperature on the larvicidal efficacy of strains

Temperature range of 25-30°C was found to be suitable for cultivation with the best efficacy all Ethiopian strains (Fig. 3). However, relatively wider range of temperature (25-35°C) was found to be suitable for the toxicity of *Bacillus* NA-71. All strains commonly exhibited maximum toxicity when they were cultivated at 30°C. As temperature of incubation was raised above 30°C, the efficacy of all strains started to decrease. However, the degree in the reduction of toxicity was variable among all strains. All strains were low in their larvicidal efficacy when they were harvested from cultures incubated at 40°C.

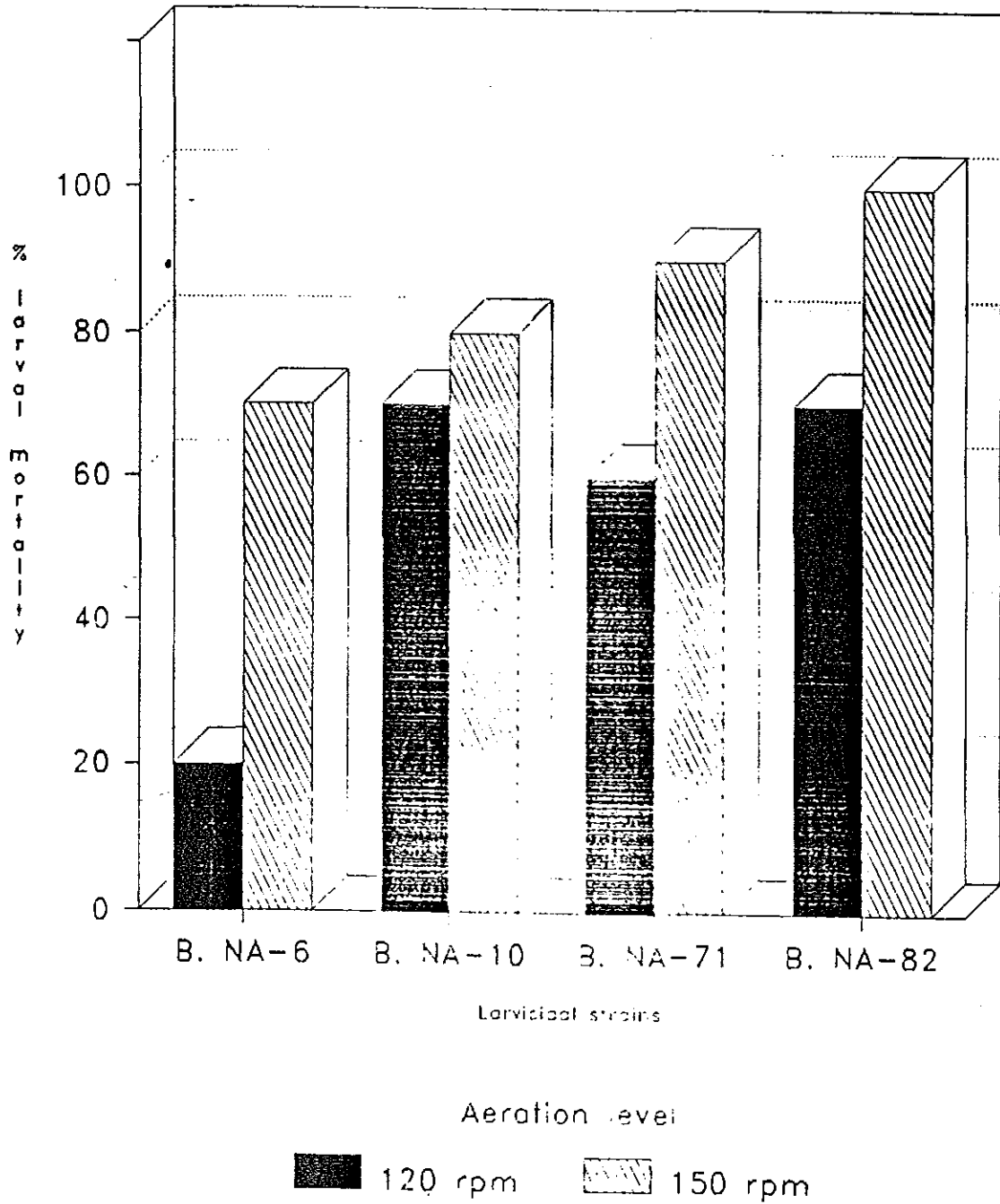
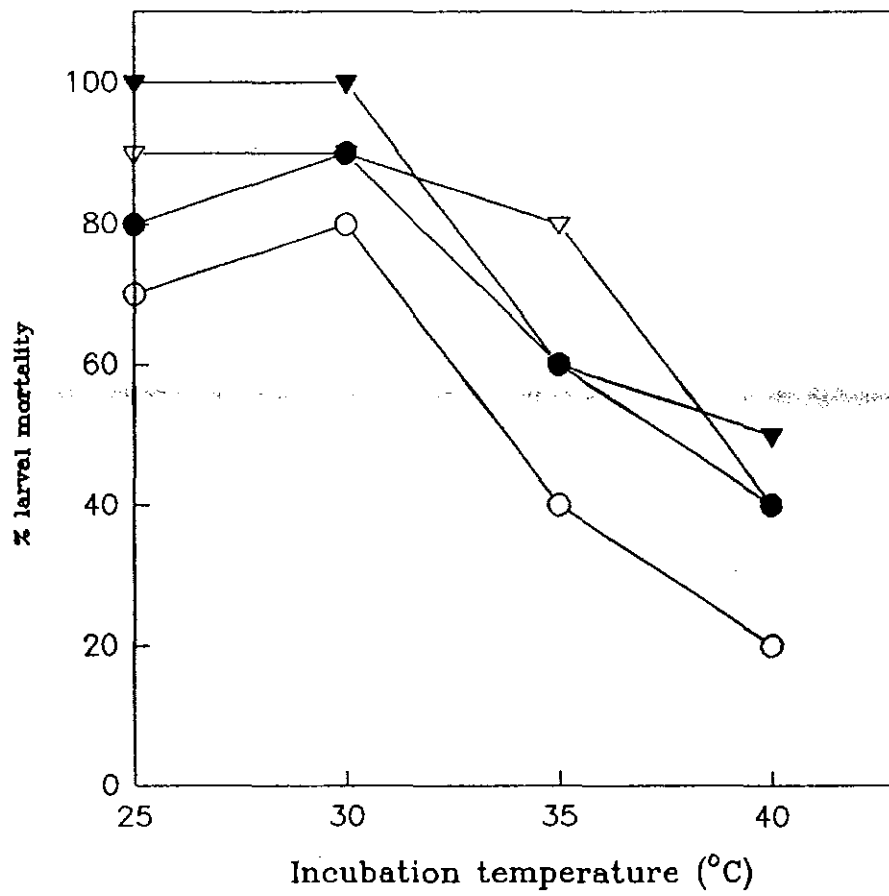


Fig.2. Effect of aeration on the larvicidal efficacy of strains.



Larvicidal strains

- B. NA-6
- B. NA-10
- ▽ B. NA-71
- ▼ B. NA-82

Fig.3. Effect of culture incubation temperature on the larvicidal efficacy of strains.

#### 4.2.4 Sporulation and crystal toxin formation at optimal conditions

The optimal media types and culture conditions of most strains resulted in a very high rate of sporulation. However, the degree of toxic crystal protein formation varied among all strains (Table 2). *Bacillus* NA-82 showed the highest level of crystal formation, followed by *Bacillus* NA-10 and *Bacillus* NA-7. Although *Bacillus* NA-6 yielded very high sporulation, it was found to be the lowest in toxic crystal protein formation.

Table 2. Relative degree of sporulation and crystal formation at optimal culture

### 4.3 Efficacy of strains on larvae of *Culex quinquefasciatus* & *Anopheles arabiensis*

Differences in the efficacy,  $LC_{50}$  values at 48 hr of the two standard and the Ethiopian strains against the different larval instars of *Cx. quinquefasciatus* (Table 3) and *An. arabiensis* (Table 4) were observed. All the strains commonly exhibited relatively high efficacy on 2<sup>nd</sup> instar and followed by 3<sup>rd</sup> and 4<sup>th</sup> instar in decreasing order.

Moreover, differences were also observed on the efficacy of each strain on the same instar levels of the two mosquito species. *Bacillus* NA-10, *Bacillus* NA-71 and IPS-82 showed higher efficacy on larvae of *An. arabiensis* than on the same instar

ers of *Cx. quinquefasciatus*.

95% confidence limit		Heterogeneity ( $\chi^2$ 3, d.f)
Lower	Upper	
1	0.0018	4.7
10	0.0043	1.0
12	0.0109	3.08
17	0.0015	0.2864
21	0.0046	0.5795
26	0.0076	0.3302
27	0.0113	0.07
31	0.0620	4.24
34	0.0623	2.52
	26.90	0.51
3	26.05	0.08
7	32.15	3.19
7	49.64	2.52
9	75.12	1.26
4	72.4	0.9043
4	117.8	4.08
5	285.7	1.79

1 3<sup>rd</sup> instars larvae of *An. arabiensis*.

95% confidence limits		Heterogeneity ( $X^2$ , 3 d.f.)
lower	upper	
0.0007	0.0013	5.80
0.0014	0.0023	0.4383
0.0022	0.0049	1.10
0.0049	0.0101	0.9744
0.0203	0.0463	4.45
0.0722	0.1292	8.8
2.54	6.41	3.05
0.2728	11.61	0.5046
7.59	23.29	6.53
9.38	23.65	0.6279
59.90	131.1	2.71
131.4	854.3	2.12

#### 4.4 Larvicidal activity in natural water

*Bacillus* NA-82 and the two standard strains were effective against field collected mosquito larval populations of *Culex* species. IPS-82 and SPH-88 were found to have better efficacy than the most effective of the Ethiopian strain, *Bacillus* NA-82 (Table 5). IPS-82 at 1 mg/10 lit gave an excellent kill (94.7% reduction) in 24 hr and complete kill in 48 hr.

The water which was obtained from the natural breeding source, Kebena river in Addis Ababa, was slightly turbid with optical density ranging from 0.03 - 0.24 A at 600 nm, the pH ranging from 7.22 - 8.65 and the day time water temperature at

Table 5. Larvicidal activity of the two standard strains and *Bacillus* NA-82 on *Culex* larvae in natural water.

Code of strains	Amount of test materials (mg/10 lit)	No. of mosquit larvae	Mean No. of mosquito larvae dead (percent. reduction)		P-values
			24 hr	48 hr	
IPS-82 Control	1.0 -	200 200	190 (94.7) 13	200 (100) 17	0.058479
IPS-82 Control	0.09 -	200 200	187 (93.2) 10	198 (98.9) 16	0.088032
IPS-82 Control	0.01	200 200	143 (69.7) 13	165 (80.8) 18	0.097865

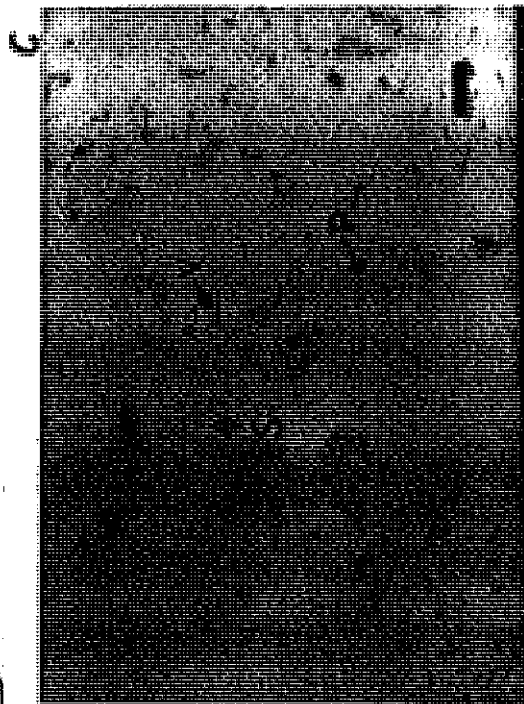
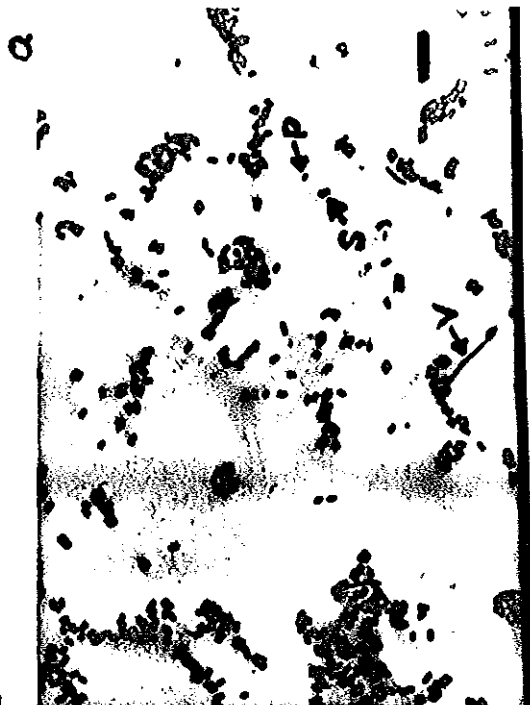
#### 4.5 Morphological and biochemical characteristics

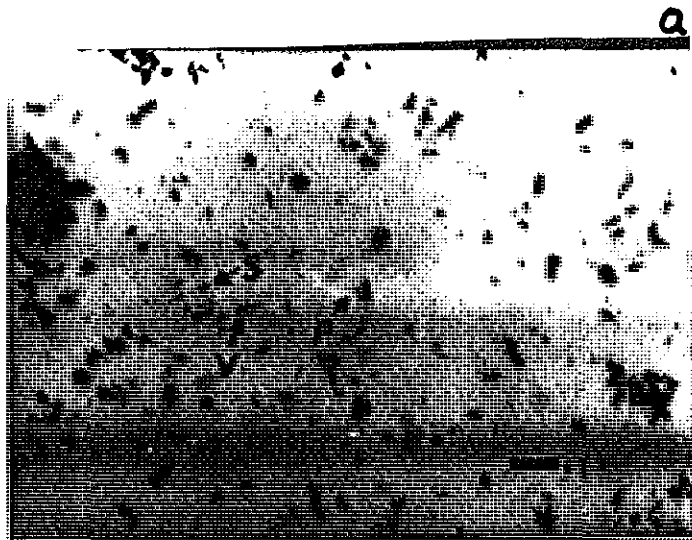
All Ethiopian strains showed typical *B. thuringiensis* morphology (Fig. 4). All the Ethiopian larvicidal strains have ellipsoidal spores with non-swollen sporangium. Moreover, all of them possess parasporal crystal proteins.

Except *Bacillus* NA-10 that showed 100% similarity (morphological & biochemical) to the reference standard strain IPS-82, all of them have some biochemical differences to this standard (Tables 6 & 7). *Bacillus* NA-6 was different from that of IPS-82 in the lack of ability to hydrolyse gelatin; *Bacillus* NA-71 was different in its ability to grow at pH less than 6 and in its ability of the deamination of phenylalanine; and *Bacillus* NA-82 was different in its ability to



Growth in NaCl	+	+	+	+	+	+
0%						
5%	+	+	+	+	+	+
7%	+	+	+	+	+	+
10%	+	+	+	+	+	+
Growth at temp.						
5°C	-	-	-	-	-	-
20°C	+	+	+	+	+	+
30°C	+	+	+	+	+	+
40°C	+	+	+	+	+	+
50°C	-	-	-	+	-	-
65°C	-	-	-	-	-	-





#### 4.6 Serological characteristics

Each of the four Ethiopian strains, *B. thuringiensis* NA-6, *B. thuringiensis* NA-10, *B. thuringiensis* NA-71 and *B. thuringiensis* NA-82 showed agglutination in slide and tube agglutination tests to two of *B. thuringiensis* H-antisera but with different degree of slide agglutinations and the agglutinin titers (Table 8). No visible agglutinations were observed in culture suspensions that were mixed with saline which served as a negative control. Culture suspension of the reference strain, *B. thuringiensis* var. *israelensis* which served as a positive control showed a very high degree of slide agglutination and also found positive for the tube

Table 8. H- agglutination of the four mosquito toxic *B. thuringiensis* strains from Ethiopia.

Code of strains	Type antisera positive with slide & tube tests	Degree of slide agglutination	The agglutinin titer of the tube test
<i>B.t.</i> NA-6	<i>B.t. seoulensis</i>	++++	102,400
	<i>B.t. neoleonensis</i>	++	1,600
<i>B.t.</i> NA-10	<i>B.t. neoleonensis</i>	++	800
	<i>B.t. morrisoni</i>	+	400

## 5 DISCUSSION

Preliminary evaluation on the toxicity of a new strain of *Bacillus* species against mosquito larvae is essential to decide whether or not the strain is worthy of further testing and evaluation. According to the protocol for the isolation and screening of spore-forming bacteria possessing vector control potential, if larval mortality is 40% or greater in 48 hr, the test strain should be considered worthy of further study and evaluation (WHO, 1987). Based on the above criteria, four of the Ethiopian strains, *Bacillus* NA-6, *Bacillus* NA-10, *Bacillus* NA-71 and *Bacillus* NA-82 fulfilled the minimum requirement (Table 1) for the primary evaluation procedure that has been

The toxicity levels produced by each of the strains were different among the media types used for cultivation of the strains (Fig. 1). Thus, the composition of the medium influences toxin production. In order to determine which part of the medium is very essential for toxin production, further investigation is required on the individual effects of the major ingredients present in each medium.

Investigation on the effect of temperature and aeration levels of culture preparations for the larvicidal activity of the Ethiopian strains (Figs. 2-3) revealed that, temperature ranges of 25-30°C and aeration level of 150 rpm were suitable for toxin production by all strains. The effect of temperature and oxygen levels on mosquito larval toxin formation by *B. sphaericus* (Yousten *et al.*, 1984) and *B. thuringiensis* H-14

was observed on the degree of sporulation and crystal formation. As a result the degree of sporulation can not be indicative for the larvicidal potential of the strains. Very little is known about the mechanisms involved in the initiation of crystal formation and in the linkage of crystal formation to sporulation (Kim *et al.*, 1994).

As shown in Tables 3 and 4 both the standard and Ethiopian strains exhibited relatively high level of efficacy on early larval instars than older instars of the two mosquito species used. This finding is in agreement with that reported by Mulla *et al.* (1980), Wraight *et al.* (1981) and Levy *et al.* (1986). Therefore, early application of microbial larvicides is required for effective control of mosquito larvae.

A recent review by Porter *et al.* (1993) concluded that in general species of

different strains of *B. thuringiensis* differ both in the potency of their toxin and the spectrum of activity toward a variety of insects (Dulmage, 1981). Baumann *et al.* (1991) have also described that the differences in the susceptibility of the mosquito larvae to the toxin proteins are in part due to differences in the binding of the toxin proteins to the target sites. The crystal toxins require a specific plasma membrane receptors on the mid gut epithelial cells (Van Rie *et al.*, 1990).

Moreover, the results obtained from the laboratory bioassay of the two Ethiopian strains and IPS-82 required extensive field trials to determine whether or not these strains also are more effective against larvae of *An. arabiensis* than on *Cx. quinquefasciatus* in the field. *An. arabiensis* is an important malaria vector and is the

this strain was much better than those reported for some other formulations of *thuringiensis* H-14. For example, Nugud and White (1982) reported  $LC_{50}$  values 2.82, 2.97 and 0.64 mg/l after 48 hr exposure for IPS-78 (the first international standard of *B. thuringiensis* H-14), Abbott and Sandoz formulations of *B. thuringiensis* H-14, respectively on second instars larvae of *An. arabiensis*. Therefore, in view of the efficacy reported for those formulations of larvicidal bacilli, *Bacillus* NA-82 was a promising candidate microbial control agent and was worthy of further development and small scale production for preliminary field trials.

In contrast, the larvicidal efficacy of *Bacillus* NA-6, *Bacillus* NA-10 and *Bacillus* NA-71 were lower than those reported for other formulations of *B. thuringiensis* H-14.

Strains with minor insecticidal activity for mosquito larvae may be important in the control of other vector insects (Weiser, 1991). Moreover, Hall *et al.* (1977) claimed that some lepidopteran-toxic strains of *B. thuringiensis* were toxic to mosquito larvae (Diptera), but the effect has little or no practical value. It is, therefore, of considerable practical importance to evaluate their efficacy against larvae of different insect vectors of disease and agricultural pests.

The larvicidal efficacies obtained from the two international standard strains against larvae of *Cx. quinquefasciatus* did not show significant differences ( $p < 0.05$ ). However, IPS-82 showed significantly higher efficacy than SPH-88 against larvae of *An. arabiensis* ( $p < 0.05$ ).

hr and there were no significant differences between the 24 and 48 hr reductions of larval populations ( $P > 0.05$ ). This has been due to the rapid larvicidal action of IPS-82. Larval death due to *B. thuringiensis* var. *israelensis* is immediate and probably due to a quick acting toxin (Ignoffo *et al.*, 1980). The differences in the 24 and 48 hr reduction of larval populations by SPH-88 and *Bacillus* NA-82 were significant ( $P < 0.001$ ). Therefore, unlike the standard IPS-82 these two larvicidal strains were slow in their larvicidal activities.

The wide variations in environmental conditions, such as water temperature, pH and turbidity made comparisons difficult with the reported larvicidal activity of *B. thuringiensis* H-14 and *B. sphaericus* in the natural environment. Darwazeh *et al.* (1990) have shown that several environmental factors in mosquito breeding habitats

described in the Bergey's Manual of Determinative Bacteriology (Claus and Berkeley 1986) only strains of *B. popilliae* and *B. thuringiensis* are known to produce parasporal crystals. In addition, recent findings have shown that highly toxic mosquito strains of *B. sphaericus* also produce toxic crystals which are protected with an exosporium (Baumann *et al.*, 1991; Porter *et al.*, 1993).

Unlike the Ethiopian strains, *B. popilliae* is characterized by the swollen sporangia, and by the negative reactions to the production of catalase, hydrolysis of casein and starch, utilization of citrate, reduction of nitrate to nitrite and growth on media with 5%, 7%, and 10% NaCl (Claus and Berkeley, 1986). Therefore, Ethiopian strains are different in those major characteristics and can not be classified

Among the four mosquito toxic *B. thuringiensis* strains examined serologically, *B. thuringiensis* NA-6 and *B. thuringiensis* NA-71 were identical to *B. thuringiensis* var. *seoulensis* with the agglutinin titer of 102,400 (Table 8). Both strains also showed agglutination to anti-*B. thuringiensis* var. *neoleonensis* serum but with a relatively low degree of slide agglutination and the agglutinin titers. H-agglutination of Ethiopian strains to two of the H-antisera of *B. thuringiensis* revealed the presence of common antigens or H-antigenic sub factors in *B. thuringiensis* serotypes. Ohba and Aizawa (1979) have also shown the presence of H-antigenic sub factors in *B. thuringiensis* var. *toumanoffi* and *B. thuringiensis* var. *kyushuensis*.

Although *B. thuringiensis* var. *seoulensis* is one of the 45 known varieties of

communication). The very low titer obtained from *B. thuringiensis* NA-10 and *B. thuringiensis* NA-82 with the type antisera of the already known serotypes may be due to a new H-sub antigenic fraction of these strains. Therefore, it is necessary to use the cross-saturation method before designating new H-antigen pattern for these strains.

Lee *et al.* (1994) have described the possible appearance of novel mosquito toxins in the new mosquitocidal strains. The identification of novel toxins from these strains will increase the diversity of mosquitocidal toxins and will help to diversify the continued use of bacterial toxins for future mosquito control. Therefore, the characterization of mosquitocidal toxins of the two Ethiopian strains, *B. thuringiensis*

## 6 RECOMMENDATIONS

1. The study showed that *B. thuringiensis* is a common bacterium in soil in the body of dead mosquito larvae in nature. They could be further sources for wild strains with desirable characteristics for the control of mosquito vector disease.

2. The type of crystal proteins (delta endotoxins) produced by the strains from Ethiopia should be characterized to determine the similarities and or the differences to the toxins produced by known larvicidal *B. thuringiensis* serotypes.

3. Attempts should be made to develop appropriate technology for local

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